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CREB-dependent Nur77 induction following depolarization in PC12 cells and neurons is modulated by MEF2 transcription factors

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Abstract

Expression of the nuclear orphan receptor gene Nur77 in neuronal cells is induced by activity-dependent increases in intracellular Ca^{2+} ions. Ca^{2+} responsiveness of the Nur77 gene has been attributed to two distinct DNA regulatory regions that recruit the transcription factors cAMP response element binding protein (CREB) and myocyte enhancer factor-2 (MEF2). Here we used dominant interfering and constitutively active mutants of CREB and MEF2 proteins to assess their relative contribution to depolarization-induced Nur77 expression in undifferentiated PC12 cells and hippocampal neurons. We show that while CREB is necessary for Ca^{2+}-activated Nur77 expression MEF2 functions to modulate CREB-dependent Nur77 expression by acting as a repressor in quiescent cells.

Keywords: cAMP response element binding protein, myocyte enhancer factor-2, neurons, Nur77, PC12 cells, Transcriptional Regulation.

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Nur77 (NR4A1) belongs to the NR4A subfamily of nuclear orphan receptors that comprise of Nur77 (NR4A1), Nurr1 (NR4A2) and Nor-1 (NR4A3) and that act as stimulus-induced transcriptional activators in many tissues including skeletal and cardiac muscle, T-cells, liver and brain (reviewed in Maxwell and Muscat 2006). Since its discovery as a gene induced by nerve growth factor in PC12 cells (Milbrandt 1988; Yoon and Lau 1993), Nur77 expression has been documented in neurons and neuronal cell lines where it is induced by growth factors (Kendall *et al.* 1994; Dickey *et al.* 2004) and synaptic activity (Shalizi *et al.* 2006; Zhang *et al.* 2007). Recent work has shown that activity-dependent changes in Nur77 expression play an important role in the dendritic development of cerebellar granule neurons where repression of Nur77 is required for synapse formation (Shalizi *et al.* 2006).

In PC12 cells Nur77 expression is induced by membrane depolarization through increases in intracellular Ca^{2+} and the subsequent activation of Ca^{2+}/calmodulin dependent protein kinases and the Ca^{2+}/calmodulin-activated phosphatase calcineurin (Yoon and Lau 1993, 1994; Enslen and Soderling 1994) and does not require activation of the MAPK signalling pathway (Machado *et al.* 2008). Two Ca^{2+}-activated transcription factors that are downstream targets of calcineurin and calmodulin dependent protein kinases and that have been shown to regulate Nur77 expression are the basic leucine zipper containing transcription factor cAMP response element binding protein (CREB) and myocyte enhancer factor-2 (MEF2), which belongs to the MCM1-Agamous-Deficiens-SRF (MADS) box family of transcription factors. The Nur77 gene contains four copies of a CRE that binds CREB and two binding sites for MEF2 factors.

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Abbreviations used: CREB, cAMP response element binding protein; GFP, green fluorescent protein; HDACs, histone deacetylases; MADS, MCM1-Agamous-Deficiens-SRF; MEF2, myocyte enhancer factor-2; MEF2DN, MEF2 dominant negative construct; MEF2-EN, MEF2-Engrailed; MRE, MEF2 response element; NFAT, nuclear factor of activated T-cells; PBS, phosphate-buffered saline; SDS, sodium dodecyl sulfate; SUMO, small ubiquitin-related modifier.

The Nur77 CREs (TGCGTCA) diverge from the consensus CRE sequence of TGACGTCA and have previously been defined as AP1 (activator protein 1) elements (Yoon and Lau 1993). Early work by Yoon and Lau (1994) in fact implicated JunD, and not CREB, in mediating Nur77 transcription through these AP1-like elements. More recently, Fass *et al.* (2003) identified Nur77 as a CREB target gene in PC12 cells by expressing a constitutively active mutant of CREB and screening for genes whose expression was up-regulated. CREB occupancy of the Nur77 promoter was further confirmed by serial analysis of chromatin occupancy using an antibody against CREB to immunoprecipitate chromatin (Impey *et al.* 2004). The MEF2 response elements (MRE) of the Nur77 gene were first identified as Ca^{2+}-responsive elements in T-cells where the MEF2 sites are required for optimal Nur77 induction by Ca^{2+}-mobilizing stimuli (Woronicz *et al.* 1995). Recently, Shalizi *et al.* (2006) used chromatin immunoprecipitation assays to show that the Nur77 promoter was also occupied by MEF2 proteins in neurons. Furthermore, a dominant repressor form of MEF2A repressed expression of a luciferase reporter gene driven by the MEF2 binding sites of Nur77 suggesting that MEF2 proteins may be important for Nur77 expression. However, early work done in PC12 cells showed that deletion of the MRE containing region of the Nur77 gene did not affect its expression following membrane depolarization, suggesting that the MREs are not essential for Ca^{2+}-induced Nur77 expression in these cells (Yoon and Lau 1993). As Shalizi *et al.* (2006) tested the effects of engineered MEF2 repressors on a reporter gene that is driven only by the Nur77 MREs, it raises questions about the contribution of MEF2 proteins to Ca^{2+}-activated Nur77 expression in neurons and neuronal cell lines. To resolve this, here we assessed the effects of MEF2 and CREB dominant interfering and constitutively active mutants on Ca^{2+}-activated expression of a Nur77 reporter gene driven by both the MREs and CREs.

Experimental procedures

Plasmids

The MEF2 dominant negative construct (MEF2DN) encoding the myc-tagged conserved MADS-box/MEF2 domain of MEF2D was constructed by cloning a PCR amplified fragment of MEF2D corresponding to amino acids 1–87 between the *Kpn*1 and *Bam*H1 sites of pcDNA3.1-myc-His (Invitrogen, Carlsbad, CA, USA). Constitutively active MEF2 was generated by cloning the transcriptional activation domain of VP16 (amplified from plasmid Gal4VP16) into the MEF2DN plasmid between the *Bam*H1 and *Not*1 sites. The expression plasmid for dominant negative A-CREB was a gift from Prof. David Ginty (The John Hopkins School of Medicine, Baltimore, MD, USA). VP16CREB expression vector (Zhang and Bading, unpublished data) was kindly provided by Prof. Hilmar Bading (University of Heidelberg, Germany). The Nur77 luciferase reporter constructs −1800Nur77Luc, −250Nur77Luc and −316/−252Nur77Luc have been described in Blaeser *et al.* (2000) and were kindly provided by Prof. Talal Chatila (UCLA, CA). phRL-SV40 expressing Renilla luciferase was from Promega (Madison, WI, USA). Plasmid MEF2-EN, expressing MEF2-engrailed fusion protein, was provided by Prof. Ilona Skerjanc (University of Ottawa, Canada) and MEF2DK439A was provided by Dr Xiang-Jiao Yang (McGill University Health Center, Montreal, Canada). The expression plasmid encoding green fluorescent protein (GFP) coupled-nuclear factor of activated T-cells (NFAT4) (pcDNA3-GFP-NFAT4) was kindly provided by Prof. Frank McKeon (Harvard Medical School, MA, USA) and the NFAT inhibitor GFP-VIVIT (Aramburu *et al.* 1999) was obtained from Prof. Anjana Rao (Harvard Medical School, MA, USA).

Cell culture and luciferase assays

PC12 cells were maintained in Dulbecco's modified Eagle's medium containing GlutaMAX I, 4.5 g/L glucose and 110 mg/L pyruvate (Invitrogen), 100 U/mL penicillin, 100 μg/mL streptomycin (Invitrogen) supplemented with 10% heat-inactivated horse serum (Invitrogen) and 5% fetal calf serum (PAA Lab, Pasching, Austria). Hippocampal neurons were cultured and transfected as described in Lam *et al.* (2009). PC12 cells and hippocampal neurons cultured in 12-well poly-D-lysine coated plates were transfected with the indicated plasmids using Lipofectamine 2000 (Invitrogen). Thirty-six hours after transfection cells were either left untreated or stimulated for 6 h as indicated and then subjected to luciferase assays as described previously (Belfield *et al.* 2006) using the Promega Dual Glo assay kit (Promega). Firefly luciferase activity was normalized to the Renilla luciferase signal and all measurements were made in duplicate.

Immunoblotting

For western blots total proteins were extracted by lyzing cells with radio immunoprecipitation assay (RIPA) buffer containing 50 mM Tris, 150 mM NaCl, 1 mM EDTA, 1% Sodium deoxycholate, 1% Triton X100, 0.1% sodium dodecyl sulfate (SDS) and protease inhibitor cocktail (Calbiochem, Merck, Darmstadt, Germany). Protein concentration was determined using protein assay DC kit (Bio-Rad, Hercules, CA, USA) according to the manufacturer's instruction. Fifty μg protein lysate was loaded onto a pre-cast 12% Novex Tris–glycine polyacrylamide gel (Invitrogen) followed by electrophoretic separation at 150 V for 1.5 h in buffer containing 25 mM Tris, 192 mM glycine and 0.1% SDS. The proteins were then transferred to polyvinylidene difluoride membrane at 30 V for 2.5 h at 4°C in transfer buffer containing 25 mM Tris, 192 mM glycine, 0.1% SDS and 20% methanol. The polyvinylidene difluoride membrane was incubated with blocking buffer containing 5% skimmed milk in Tris buffered saline with Tween (TBST) for 1 h, followed by incubation with primary antibody diluted in blocking buffer overnight at 4°C. The following antibodies were used at the indicated dilutions: MEF2D specific antibody (BD Pharmingen, Franklin Lakes, NJ, USA) at 1 : 2000; phospho-CREB at 1 : 5000 (Upstate, Lake Placid, NY, USA); β-actin at 1 : 10 000 (Sigma, St Louis, MO, USA). After removal of primary antibody, the membrane was rinsed three times (10 min each) and incubated with horseradish peroxidase (HRP) conjugated secondary antibodies (DAKO, Glostrup, Denmark) at 1 : 1000 dilution for 1 h at 25°C. The membranes were then washed three times (10 min each) and the

bands were visualized using ECL detection kit (Amersham, Little Chafont, Buckinghamshire, UK).

NFAT localization

To assess NFAT localization PC12 cells grown on 13 mm coverslips were transfected with an expression plasmid encoding GFP-NFAT4. Twenty-four hours after transfection cells were stimulated with 50 mM KCl for 45 min or left untreated. Cells were fixed in 3% paraformaldehyde in phosphate-buffered saline (PBS) containing 4% sucrose as described earlier (Lam and Chawla 2007). Cells were then washed twice with 10 mM glycine in PBS for 10 min, and counter-stained with Hoechst 33342 (1 μg/mL in PBS; Invitrogen) for 5 min to visualize nuclei. Cells were washed twice in with PBS to remove excess Hoechst dye and mounted in Vectashield (Vector Laboratories, Burlingame, CA, USA). GFP and Hoechst fluorescence was detected using an inverted Leica TCS SP5 confocal system (Leica Microsystems, Wetzlar, Germany) and a 20× water immersion objective lens.

Results

We used a luciferase reporter construct that contains the firefly luciferase gene downstream of rat Nur77 genomic DNA from −1800 to +119 bp relative to the transcription start site. Figure 1(a) shows the 2 MEF2 response elements (MREs) located at −309 and −275 bp upstream of the transcriptional start site and the four CREs at positions −235, −215, −71 and −42 bp relative to the transcription start site of the rat gene. The sequences are conserved in human and mouse genes. We first established that constitutively active mutants of CREB and MEF2 could activate the −1800Nur77 luciferase reporter gene (Fig. 1b). Co-expression of MEF2VP16, which encodes the MADS/MEF2 domain of mouse MEF2D (amino acids 1–87) fused to the transcriptional activation domain of VP16, potentiated −1800Nur77-Luc expression by 45.70 ± 5.38-fold relative to cells transfected with vector control. Similar to constitutively active MEF2, VP16CREB that encodes a fusion protein comprising of the VP16 activation domain and rat CREB, was also a potent activator of Nur77 expression and caused a 66.30 ± 3.16-fold increase in −1800Nur77Luc expression. This is consistent with earlier work that reported an increase in Nur77 mRNA levels by VP16CREB expression in PC12

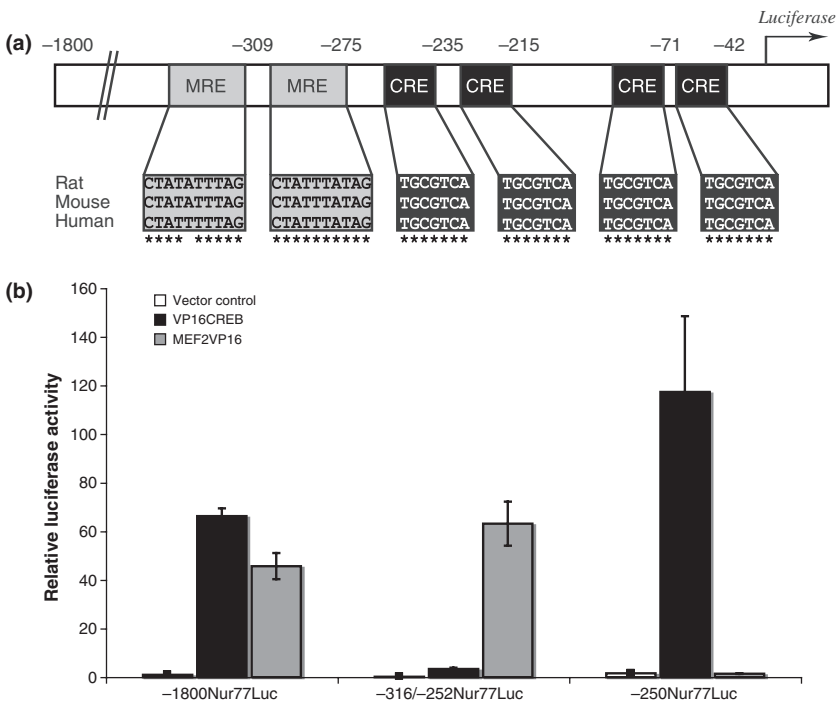

Fig. 1 The promoter of the Nur77 gene recruits CREB and MEF2 transcription factors. (a) Schematic representation of the rat Nur77 promoter region within the −1800Nur77 firefly luciferase reporter plasmid. There are four CREs at −42, −71, −215 and −235 bp upstream of the transcriptional start site (TSS). Two MREs are located at −275 and −309 bp from the TSS. The CREB and MEF2 binding sites are conserved between rat, mouse and human genes. (b) Constitutively active VP16CREB and MEF2VP16 proteins activate Nur77 transcription by binding to the corresponding CREs and MREs on the promoter. PC12 cells grown on 12-well cell culture plates were transfected with either 1 μg VP16CREB or 1 μg MEF2VP16, 500 ng of the indicated Nur77 firefly luciferase reporter plasmid (−1800Nur77Luc reporter containing the MREs and CREs, or the −316/−252Nur77Luc reporter containing only the MREs or the −250Nur77Luc reporter containing only the CREs), and 50 ng phRL-SV40 expressing renilla luciferase. Thirty-six hours after transfection, cells were lysed with passive lysis buffer and subjected to luciferase assays. Firefly luciferase activity was normalized to renilla luciferase activity. The mean ± SEM of relative luciferase activity is shown in the graph, data were generated from four independent transfection experiments. $p < 0.05$ (one-way ANOVA followed by Bonfferroni's *post-hoc* test).

cells (Fass *et al.* 2003). The effects of VP16CREB and MEF2VP16 were mediated specifically by their respective DNA binding regions of the Nur77 gene as a construct containing −316 to −252 bp of 5′ regulatory sequences, which encompasses the two MREs but lacks the CREs, was activated by MEF2VP16 only and a truncated construct containing −250 bp of the promoter that lacks the MREs was activated only by VP16CREB (Fig. 1b).

We next tested the effect of dominant negative CREB and MEF2 constructs on Nur77 induction by depolarization in PC12 cells. Membrane depolarization of PC12 cells with 50 mM extracellular KCl induced rapid activation of CREB phosphorylation on serine 133 that is necessary for CREB-mediated gene expression (Fig. 2a). Depolarization also caused a robust increase (5.38 ± 0.51-fold) in the expression of −1800Nur77 luciferase reporter gene (Fig. 2b). To assess the contribution of CREB to Nur77 induction by depolarization we interfered with CREB function by expressing A-CREB, which is an inhibitory form of CREB protein that is deficient in DNA binding but able to heterodimerize with CREB and related transcription factors such as ATF1 and cAMP responsive element modulator. A-CREB expression thus interferes with DNA binding of endogenous CREB to CREs and inhibits CREB-dependent gene expression in PC12 cells (Ahn *et al.* 1998). A-CREB inhibited induction of −1800Nur77 luciferase by depolarization and also attenuated basal expression of the Nur77 reporter gene (Fig. 2b). To assess the contribution of MEF2 factors to Nur77 expression we expressed a dominant negative MEF2 protein, MEF2DN that encodes the MADS/MEF2 DNA binding domain of MEF2D and lacks the C-terminal region that contains the transactivation domain. The MADS/MEF2 domain is conserved amongst the four MEF2 family members MEF2A, MEF2B, MEF2C and MEF2D, and recruits transcriptional repressors such as Cabin1/Cain, Class IIa histone deacetylases (HDACs) and MEF2-interacting transcriptional repressor (MITR) (Kim *et al.* 2005 and references therein) that dissociate from MEF2 proteins during their Ca^{2+}-dependent activation. Expression of the MADS/MEF2 domain of MEF2A has been shown previously to act in a dominant negative manner in muscle cells where it blocked myogenic differentiation and inhibited expression of myogenin and myosin heavy chain genes (Ornatsky *et al.* 1997) and in P19 cells where it induced apoptosis and inhibited neurogenesis (Okamoto *et al.* 2000). Contrary to our expectations, the dominant negative MEF2 inhibitor MEF2DN did not inhibit Nur77 induction by depolarization but in fact augmented it (Fig. 2b). In the presence of MEF2DN Nur77 induction by depolarization was more than doubled to 213.97 ± 28.35% of the KCl response in the presence of a vector control plasmid (Fig. 2b). MEF2DN expression also increased basal Nur77 expression in the absence of depolarization by 94.12 ± 24.53% (Fig. 2b). To rule out the possibility that MEF2DN is unable to act in a dominant negative manner

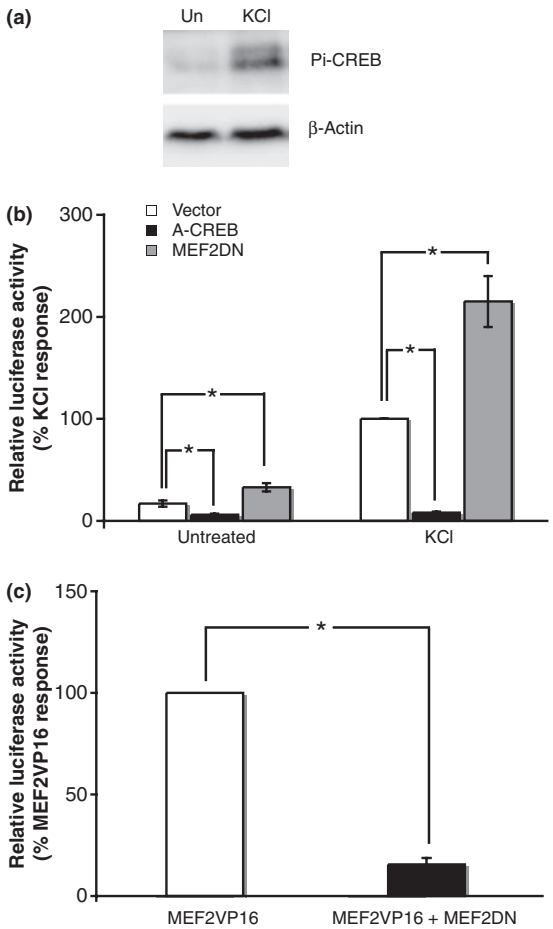

Fig. 2 Depolarization-induced Nur77 expression in PC12 cells is regulated by CREB and modulated by MEF2. (a) Membrane depolarization of PC12 cells induces CREB phosphorylation on serine 133. PC12 cells grown on 35 mm dishes were left untreated (Un) or stimulated with 50 mM extracellular KCl (KCl) for 20 min and processed for immunoblotting using an antibody that recognizes CREB phosphorylated on serine 133 (top panel). Blots were then stripped and probed with an antibody towards β-actin to control for protein loading (bottom panel). (b) −1800Nur77Luc expression is inhibited by a dominant interfering mutant of CREB (A-CREB) but enhanced by dominant interfering form of MEF2 (MEF2DN). PC12 cells grown on 12-well cell culture plates were transfected with 500 ng −1800Nur77Luc, 50 ng phRL-SV40 and 1 μg of the indicated dominant negative CREB or MEF2 expression vector. Thirty-six hours after transfection, cells were depolarized with 50 mM extracellular KCl for 6 h, and then lysed for luciferase assays. Firefly luciferase activity was normalized to renilla luciferase activity. The mean ± SEM of relative luciferase activity is shown in the graph as a percentage of the KCl response, data were generated from four independent transfection experiments. (c) MEF2DN inhibits the activation of −1800Nur77Luc induction by constitutively active MEF2 (MEF2VP16). PC12 cells were transfected with 1 μg MEF2VP16, 500 ng −1800Nur77Luc, 50 ng phRL-SV40 and 1 μg of either the vector or MEF2DN expression plasmid. Luciferase activity was determined 36 h after transfection as described for (b). Data shown are from six independent experiments. *$p < 0.05$ (one-way ANOVA followed by Bonfferroni's *post-hoc* test).

because of an inability to bind DNA and compete with endogenous MEF2 factors, we tested the effect of MEF2DN on MEF2VP16-mediated increases in Nur77 expression. Figure 2(c) shows that co-expression of MEF2DN reduced MEF2VP16 induced −1800Nur77Luc expression to 11.78 ± 3.30%. Thus, MEF2DN inhibits the ability of MEF2VP16 to activate −1800Nur77Luc expression.

The lack of inhibition by MEF2DN may be explained by the fact that although it can recruit transcriptional repressors and does not have the C-terminal transactivation domain it also lacks the region of MEF2 that is controlled negatively by a post-translational modification with small ubiquitin-related modifier (SUMO). Sumoylation of MEF2A, C and D has been documented and interferes with the transcriptional activity of all three isoforms (Gregoire *et al.* 2006; Kang *et al.* 2006; Shalizi *et al.* 2006). We hypothesized that MEF2DN prevents binding of endogenous MEF2s that can be sumoylated and is therefore competing for the repressive effects of MEF2 on the Nur77 promoter. To test this hypothesis we assessed the effects of a SUMO-resistant mutant of MEF2D where the lysine residue at position 439 that would normally be sumoylated is mutated to an alanine. Figure 3(a) shows that a MEF2DK439A mutant acts in a similar fashion to MEF2DN in augmenting basal and depolarization-induced expression of −1800Nur77Luc. MEF2DN and the SUMO-resistant mutant MEF2DK439A also had similar effects on VP16CREB-induced expression of Nur77 (Fig. 3b). Sumoylation of MEF2D on lysine 439 requires its phosphorylation on serine 444 (Gregoire *et al.* 2006). Increases in intracellular Ca^{2+} induce a calcineurin-dependent dephosphorylation of serine 444 to promote a switch from sumoylation to acetylation on lysine 439, resulting in a relief of sumoylation-mediated repression of MEF2D. Western blot analysis of MEF2D in unstimulated and depolarized PC12 cells revealed that depolarization alters the mobility of MEF2D in a manner that would be consistent with its dephosphorylation (Fig. 3c, compare lanes 1 and 2 and lanes 4 and 5). The change in MEF2D mobility was observed only after depolarization and not by nerve growth factor stimulation of PC12 cells and was sensitive to the calcineurin inhibitor cyclosporine A (Fig. 3c). This suggests that in unstimulated PC12 cells MEF2D is likely to be sumoylated. We next assessed what consequences a repressor form of MEF2, such as a sumoylated MEF2 protein, would have for Nur77 expression when recruited to the Nur77 MEF2 binding sites. We used a mutant MEF2 protein in

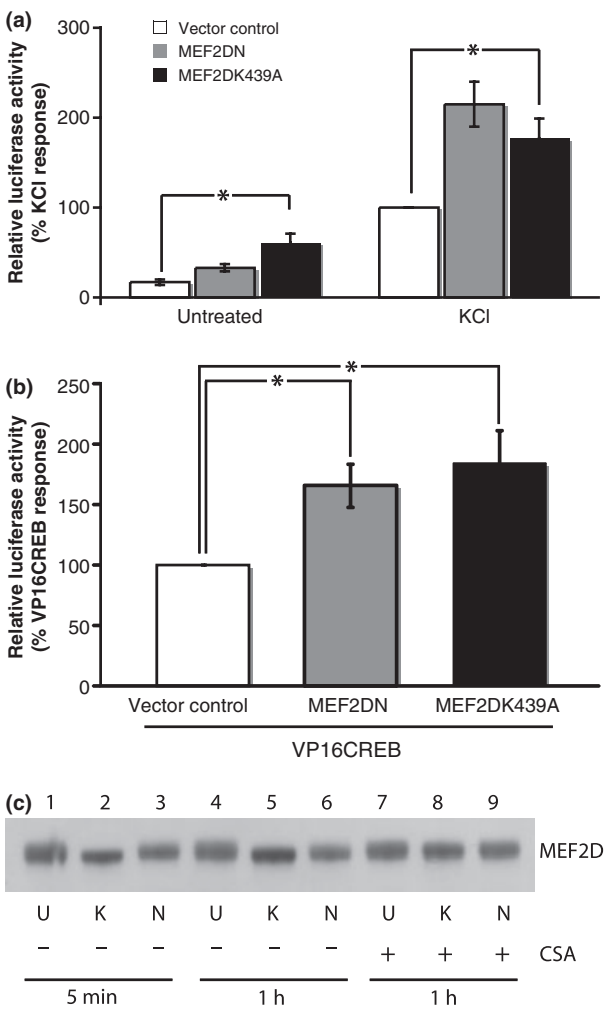

Fig. 3 MEF2 proteins act as repressors through the C-terminal domain. (a) The introduction of a sumoylation resistant mutant of MEF2D (MEF2DK439A) enhances Nur77 expression to a similar extent as MEF2DN in untreated or depolarized cells. PC12 cells grown on 12-well cell culture plates were transfected with 500 ng −1800Nur77Luc, 50 ng phRL-SV40 and 1 μg of either MEF2DN or MEF2K439A. Thirty-six hours after transfection cells were depolarized and lysed for luciferase assays as described in Fig. 2. Data shown in the graph are from four independent transfection experiments. (b) MEF2K439A and MEF2DN augment Nur77 expression induced by constitutively active CREB (VP16CREB). PC12 cells were transfected with 500 ng −1800Nur77Luc, 50 ng phRL-SV40 and 1 μg VP16CREB and 1 μg of either MEF2DN or MEF2K439A expression plasmid. Luciferase activity was determined 36 h after transfection. The mean ± SEM of relative luciferase activity is shown in the graph, data were generated from three independent experiments. $*p < 0.05$ (one-way ANOVA followed by Bonfferroni's *post-hoc* test). (c) MEF2D shows a shift in mobility consistent with dephosphorylation in depolarized cells, but not in NGF treated cells (U – Untreated; K – KCl; N – NGF). MEF2D mobility shift in cells treated with KCl (compare lanes 1 and 2), but not NGF (compare lanes 1 and 3) can be detected as early as 5 min after treatment and persists for 1 h (see lanes 4–6). The mobility shift is restored by pre-treatment (10 min) with 100 nM of the calcineurin inhibitor cyclosporine A (CSA; lanes 7–9). PC12 cells cultured in 35 mm dishes were treated with 50 mM KCl or 100 ng/mL NGF, with or without 100 nM CSA as indicated. Cells were lysed and protein harvested at 5 min and 1 h after treatment. MEF2D was detected using a MEF2D-specific antibody at a dilution of 1 : 2000 (BD Pharmingen) and HRP conjugated anti-mouse IgG antibody at 1 : 1000 dilution (Dako).

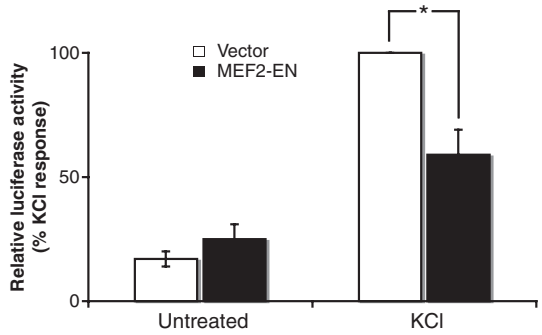

Fig. 4 Depolarization-induced Nur77 expression is inhibited by an engineered MEF2 repressor. PC12 cells grown on 12-well cell culture plates were transfected with 500 ng −1800Nur77Luc, 50 ng phRL-SV40 and 1 μg of either vector or MEF2-Engrailed (MEF2-EN) expression plasmid. Thirty-six hours after transfection, cells were depolarized with 50 mM KCl for 6 h, and then lysed for luciferase assays. The mean ± SEM of relative luciferase activity is shown in the graph, data were generated from three independent transfection experiments. *$p < 0.05$ (one-way ANOVA followed by Bonfferroni's *post-hoc* test).

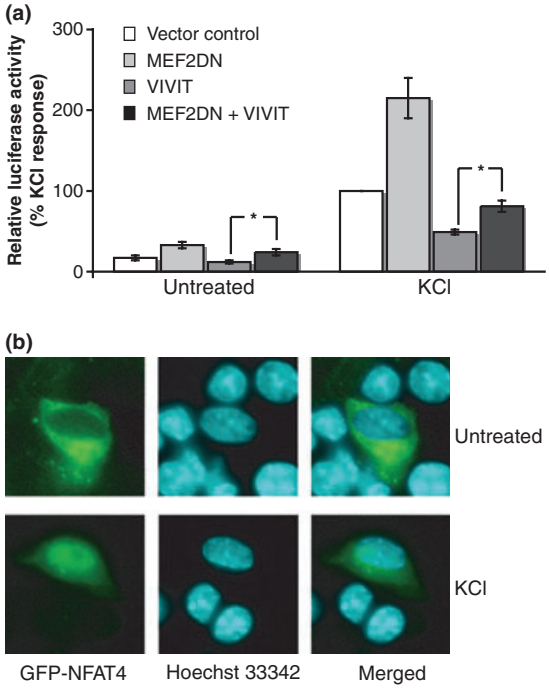

Fig. 5 MEF2DN-mediated enhancement of Nur77 expression can occur in the absence of nuclear NFAT. (a) MEF2DN rescues VIVIT-mediated inhibition of KCl-induced Nur77 expression. PC12 cells cultured in 12-well plates were transfected with −1800Nur77Luc, phRL-SV40 together with either pcDNA3.1 (vector control), VIVIT, MEF2DN or VIVIT and MEF2DN. Thirty-six hours after transfection cells were depolarized with 50 mM KCl for 6 h and the lysates subjected to luciferase assays as described for Fig. 2. Data were generated from three independent transfection experiments. *$p < 0.05$ (one-way ANOVA followed by Bonfferroni's *post-hoc* test). (b) NFAT localization is regulated by depolarization in PC12 cells. Example of cytoplasmic expression of GFP-NFAT4 (green) in unstimulated PC12 cell (upper panel), and translocation into the nucleus in the presence of KCl (lower panel). PC12 cells cultured on 13 mm coverslips were transfected with 1 μg GFP-NFAT4 and 24 h after transfection, cells were either left untreated or stimulated with 50 mM KCl for 45 min. Cells were then fixed and nuclear stained with Hoechst 33342 (Cyan).

which the MADS/MEF2 domain was fused to the transcriptional repressor Engrailed (MEF2-EN). Expression of MEF2-EN significantly attenuated (by 40.80 ± 9.89%) the induction of −1800Nur77Luc by depolarization (Fig. 4).

Our data thus far support the hypothesis that MEF2DN potentiates Nur77 expression because it contains only the MADS/MEF2 domain of MEF2D and thus is not subject to sumoylation, therefore lacking the ability to repress Nur77 transcription mediated by other transcription factors such as CREB. An alternative mechanism by which MEF2DN could increase Nur77 expression is through binding of the transcription factor NFAT, which is recruited to the Nur77 promoter through interactions with the MADS/MEF2 domain of MEF2 family members (Blaeser *et al.* 2000; Kim *et al.* 2005). Calcineurin regulates NFAT activation by dephosphorylation of NFAT proteins in the cytoplasm allowing their translocation to the nucleus. To test for the involvement of NFAT in the potentiating effects of MEF2DN we used VIVIT, a potent peptide inhibitor that interacts with the calcineurin docking site of NFAT proteins and thus disrupts binding of calcineurin to NFAT, preventing nuclear translocation of all NFAT isoforms (Aramburu *et al.* 1999). Figure 5(a) shows that in PC12 cells transfected with a plasmid encoding GFP tagged VIVIT, depolarization-induced Nur77 expression was inhibited by 51.84 ± 2.96%. This decrease in depolarization-induced Nur77 expression by VIVIT was partially rescued by co-transfection of MEF2DN (Fig. 5a, compare Nur77 expression in MEF2DN and VIVIT transfected cells with those transfected with VIVIT alone). While the decrease in the KCl-induced response by VIVIT is consistent with previous work that has implicated NFAT factors in Nur77 transcriptional induction (Blaeser *et al.* 2000), the rescue by MEF2DN in the presence of VIVIT suggests that MEF2DN is enhancing Nur77 expression by an NFAT-independent manner because NFAT is unlikely to be present in the nucleus when VIVIT is expressed. An NFAT-independent mechanism for the potentiating effects of MEF2DN is also supported by the enhancement of basal Nur77 expression by MEF2DN because under basal conditions NFAT is localized exclusively to the cytoplasm of PC12 cells (Fig. 5b, top panel). This raises questions about the transcription factor(s) mediating MEF2DN-dependent Nur77 expression under basal conditions. We tested whether this could be because of the derepression of MEF2-mediated inhibition of CREB activity under basal conditions. Figure 6(a) shows that the potentiating effect of MEF2DN

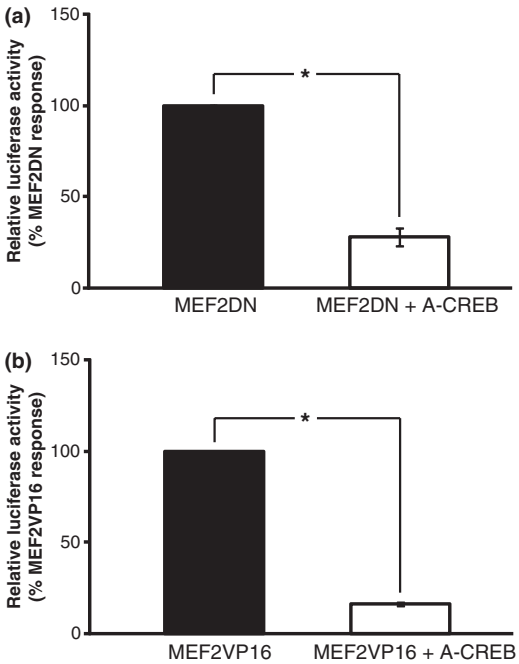

Fig. 6 MEF2DN-mediated and MEF2VP16-mediated potentiation of Nur77 expression requires CREB. (a) Co-transfection of A-CREB with MEF2DN abolished the potentiating effect of MEF2DN on Nur77 gene expression in unstimulated PC12 cells. (b) A-CREB also attenuated the activation of Nur77 gene expression mediated by MEF2VP16. PC12 cells grown on 12-well cell culture plate were co-transfected with 500 ng −1800Nur77Luc, 50 ng phRL-SV40 and 1 μg of either MEF2DN (a) or MEF2VP16 (b) with or without A-CREB. Thirty-six hours after transfection, cells were depolarized with 50 mM KCl for 6 h, and then lysed for luciferase assays. The mean ± SEM of relative luciferase activity is shown in the graph, data were generated from three independent transfection experiments. $*p < 0.05$ (one-way ANOVA followed by Bonfferroni's *post-hoc* test).

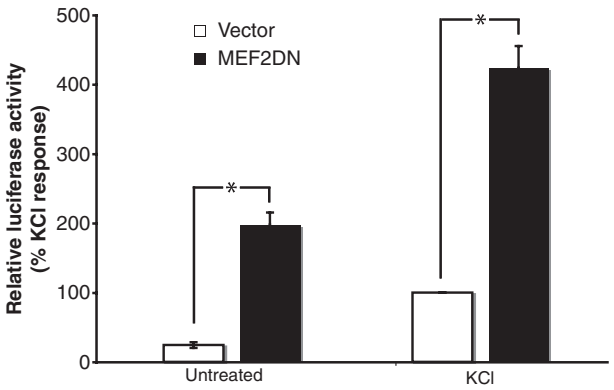

Fig. 7 MEF2DN enhances Nur77 expression in primary hippocampal neurons. MEF2DN induced Nur77 gene expression by more than eightfold in unstimulated rat hippocampal neurons. It also significantly enhanced the response induced by KCl. Primary rat hippocampal neurons were cultured on 12-well cell culture plate and were co-transfected with 1 μg MEF2DN, 500 ng −1800Nur77Luc and 50 ng phRL-SV40. Twelve hours after transfection, cells were depolarized with 40 mM KCl for 4 h, and then lysed for luciferase assays. The mean ± SEM of relative luciferase activity is shown in the graph, data were generated from three independent transfection experiments. $*p < 0.05$ (one-way ANOVA followed by Bonfferroni's *post-hoc* test).

on basal Nur77 transcription is hampered by co-expression of dominant negative CREB supporting the notion that under basal conditions MEF2 factors act to constrain CREB-mediated gene expression. Similarly, MEF2VP16-mediated Nur77 expression was also inhibited by A-CREB (Fig. 6b).

Taken together, our data suggest that in PC12 cells CREB is necessary for activation of Nur77 transcription in response to depolarization, while MEF2 proteins are required for the repression of Nur77 in quiescent cells that is relieved by Ca^{2+}-dependent signaling mechanisms when cells are stimulated. As the PC12 cells used in the experiments above are undifferentiated cells, we tested whether MEF2DN could enhance expression of the Nur77 reporter gene in terminally differentiated primary hippocampal neurons. Figure 7 shows that MEF2DN boosts expression of the Nur77 reporter in unstimulated neurons by 817.47 ± 52.01% as well as in depolarized hippocampal neurons (423.45 ± 31.73%) These findings suggest that similar to PC12 cells MEF2 factors are required for transcriptional repression of Nur77 in unstimulated hippocampal neurons.

Discussion

We have shown here that while Nur77 expression can be induced by constitutively active mutants of either CREB or MEF2 transcription factors, only a dominant interfering form of CREB, but not MEF2, abolishes Nur77 induction by membrane depolarization indicating that CREB is an essential mediator of Ca^{2+}-activated Nur77 expression in PC12 cells. The MEF2 dominant negative mutant used here encodes the MADS/MEF2 domain of mouse MEF2D that mediates DNA binding and is highly conserved amongst the four MEF2 family members and between rat, mouse and human species. MEFDN lacks the C-terminal transactivation domain, which mediates MEF2 activation in response to p38 MAPK, extracellular signal-regulated kinase 5, and extracellular signal-regulated kinase 1/2 signaling cascades (Mao *et al.* 1999; Zhao *et al.* 1999; Okamoto *et al.* 2000; Liu *et al.* 2003; Shalizi *et al.* 2003; Wang *et al.* 2007). Because of the conserved nature of the MADS/MEF2 domain the MEF2DN protein used here is expected to interfere with the function of all four MEF2 family members and the dominant interfering effect of the MADS/MEF2 domain on MEF2-mediated gene expression has previously been documented (Ornatsky *et al.* 1997). The lack of inhibition by MEF2DN on depolarization-induced Nur77 expression in PC12 cells suggests that the C-terminal domains of MEF2 transcription factors are not

necessary for Ca^{2+}-activated transcription of the gene. This is consistent with earlier work from our laboratory where we reported a robust Ca^{2+}-activated expression of a Gal4 driven luciferase reporter gene by a full length Gal4MEF2D fusion protein but not by Gal4MEF2D(C) that contains only the C-terminal domain of MEF2D (Belfield *et al.* 2006). Taken together, this suggests that the C-terminal domain of MEF2D is neither necessary nor sufficient for Ca^{2+}-activated transcription. The finding that MEF2DN enhances basal and depolarization-induced Nur77 expression is consistent with the previously reported repressive functions of MEF2 proteins that require sumoylation of the C-terminal region (Gregoire *et al.* 2006; Shalizi *et al.* 2006). However, MEF2DN would be expected to interact with transcriptional corepressors such as Cabin1 and Class IIa HDACs, HDAC4, 5 and 7 and with transcriptional activators such as NFAT that further recruits p300 (Kim *et al.* 2005). The ability of MEF2DN to enhance Nur77 expression does not however depend on the recruitment of NFAT as MEF2DN is able to potentiate Nur77 expression under conditions where NFAT is cytoplasmic in PC12 cells. The ability of MEF2DN to enhance Nur77 expression under basal conditions in PC12 cells does however depend on CREB activity as it is blocked by A-CREB, the dominant negative CREB mutant. Our finding that the ability of MEF2DN to enhance Nur77 expression is not restricted to undifferentiated PC12 cells but also occurs in terminally differentiated hippocampal neurons suggests that modulation of Nur77 expression by MEF2 factors may affect many cellular processes such as hormone secretion and differentiation that are triggered by membrane-depolarization.

Our data thus support the idea that while CREB is necessary to activate Nur77 transcription, MEF2 functions mainly as a repressor in unstimulated or non-depolarized PC12 cells and hippocampal neurons to restrain CREB-mediated expression of Nur77. This hypothesis is consistent with the finding that Nur77 expression in PC12 cells in response to Ca^{2+} signals is calcineurin sensitive (Enslen and Soderling 1994). Calcineurin controls both MEF2 and CREB transcription factors. For MEF2 factors, calcineurin activity is required for their derepression by de-sumoylation (Flavell *et al.* 2006; Gregoire *et al.* 2006; Shalizi *et al.* 2006) and for subsequent recruitment of NFAT and p300 (Youn *et al.* 2000). CREB activation is controlled by calcineurin via recruitment of the CREB coactivators transducers of regulated CREB activities that enhance CRE-dependent transcription through interactions with the basic leucine zipper DNA binding/dimerization domain of CREB (Conkright *et al.* 2003) where calcineurin activity is required for Ca^{2+}-dependent nuclear localization of transducers of regulated CREB activities (Bittinger *et al.* 2004). Many other depolarization-induced CREB target genes have functional MEF2 binding sites, and the mechanism proposed here is likely to have relevance to their regulation as well (Flavell *et al.* 2008). Examples of such genes are the NR4A family member Nor-1 (Flavell *et al.* 2008; Lam *et al.* 2009), the prototypical immediate early gene c-*fos* (Flavell *et al.* 2008; Maruthappu, and Chawla, unpublished observations), and brain derived neurotrophic factor (Flavell *et al.* 2008). We propose that MEF2 proteins may function to enhance the signal-to-noise ratio of the expression of such genes in response to Ca^{2+} signals. During basal activity MEF2 factors act as active repressors, preventing the unnecessary expression of MRE-containing genes by CREB in response to low levels of Ca^{2+}. Once Ca^{2+} levels exceed a given threshold in response to depolarization, MEF2-mediated repression is relieved and activation of transcription via CREB allows the gene to be expressed. Further work will reveal whether transcriptional repression by MEF2 proteins does enhance the signal-to-noise ratio of gene expression.

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